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13. ABSTRACT (Maximum 200) Previously, we have reported that the expression of CD44 _{v3,8-10} is closely correlated with the metastatic behavior of human breast carcinomas. In this study we have chosen a new breast cancer metastasis model system, Met-1 cells (derived from a high metastatic potential tumor in transgenic mice expressing polyomavirus middle T oncogene), to further analyze the functional property of this CD44 variant isoform. Using a variety of techniques (e.g. RT-PCR, Southern blot, immunoprecipitation and immuno-blot techniques), we have found that Met cells also express CD44 _{v3,8-10} (M.W. ≈ 260 kDa) on the cell surface. Biochemical analyses indicate that the external domain of CD44 _{v3,8-10} , which contains added heparan sulfate, preferentially binds VEGF, but not bFGF. These findings suggest that CD44 _{v3,8-10} is involved in the onset of tumor-associated angiogenesis. These cells also form "invadopodia" structures via a microfilament-dependent process. Most importantly, we have found that CD44 _{v3,8-10} is closely associated with the active form of matrix metalloproteinase, MMP-9, in a complex within the "invadopodia" structures. Therefore, we believe that CD44 _{v3,8-10} and associated molecules (e.g. VEGF and MMP-9) play an essential role in regulating metastatic tumor cell behavior during the progression of breast cancer.				
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Date 9/11/97

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(5) INTRODUCTION

CD44 isoforms belong to a family of glycoproteins which are expressed in a variety of cells and tissues (for reviews see 3,26). The external domain of certain CD44 isoforms is known to mediate both cell adhesion to extracellular matrix (ECM) components [e.g. hyaluronic acid (HA), fibronectin and collagen] and homotypic cell aggregation (26). The cytoplasmic domain of the CD44 isoforms contains a specific binding region required for ankyrin binding (3,30). Post-translational modification of CD44's cytoplasmic domain by protein kinase C (23), acylation (4) or GTP binding (29) has been found to enhance the binding between CD44 and ankyrin. These observations suggest that CD44 not only functions as an adhesion protein, but may also play an important role in signalling cytoskeleton-mediated cellular activities (3,5).

It is now known that all CD44 isoforms are encoded by a single gene which contains 19 exons (41). Out of the 19 exons, 12 exons can be alternatively spliced (1,41,45). Most often, alternative splicing occurs between exons 5 and 15 leading to an insertion in tandem of one or more variant exons within the membrane proximal region of the extracellular domain (41). The variable amino acid sequence of different CD44 isoforms is further modified by extensive N- and O-glycosylations (3,28) and glycosaminoglycan (GAG) additions (2). For example, one of the CD44 isoforms (so-called CD44v₃) contains the v3 (or exon 7) insertion which contains heparan sulfate addition sites. It has been suggested that this molecule binds a wide range of heparin binding growth factors, cytokines, and chemokines (2,21).

It is clear that the mechanisms responsible for tumor progression are very complex involving a number of biochemical and cellular events (50). In human cells, matrix metalloproteases (MMPs), such as MMP-2 (72kDa), MMP-9 (92 kDa) and MMP-3 (57kDa), are most likely involved in the initial degradation of the basement membrane surrounding the tumor (27) which is required for tumor invasion and metastasis (7,33). Recent data indicating co-localization of MMP-2 and integrin α v β 3 on the surface of invasive melanoma cells and blood vessels by

Brooks et al (8) suggest that there is a close interaction between MMPs and certain adhesion molecules (e.g. integrins) during tumor invasion and/or metastasis (8). The CD44v₃-containing isoforms, another class of adhesion molecule is also preferentially expressed on the surface of metastatic tumor cells during the progression of human breast carcinomas (6,20). The question whether there is an interaction between MMPs and CD44v₃-containing isoforms in metastatic breast tumor cells is addressed in this study.

Previously, we have reported that the expression of CD44v_{3,8-10} is closely correlated with the metastatic behavior of human breast carcinomas. In this study we have employed a unique breast cancer cell line [Met-1 which was derived from high metastatic potential tumors in transgenic mice expressing polyomavirus middle T oncogene] to study the role of CD44v₃-containing isoforms in regulating metastatic breast tumor cell behavior. Using a variety of techniques (including biochemical, immunochemical and molecular biological approaches), we have found that the CD44v_{3,8-10} isoform plays an important role in regulating metastatic tumor cell behavior including invadopodia formation, matrix degradation and angiogenesis during breast cancer progression.

(6) BODY

MATERIALS AND METHODS

Cell Culture:

Mammary tumor cells containing the polyoma virus middle T (PyV-MT) transgene under the transcriptional control of the MMTV LTR promoter were used to initiate a transplantable line in nude mice. The PyV-MT transgenic mammary tumor cells were obtained from mammary tumors which arose in the transgenic colony at the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada (Dr. William J. Muller) (17). Mammary tumors were collagenase/dispase (Worthington, Freehold, N. J.) treated, and 5×10^5 cells per 100 μ l were transplanted subcutaneously as a bolus via syringe and a 25 gauge needle into the thoracic region of nude mice. The resulting high potential metastatic PyV-MT transgenic mammary tumor line, Met-1, was maintained by serial transplantation of 1 mm³ tumor segments into either subcutaneous tissue (ectopic) or intact mammary fat pads (orthotopic).

The Met-1 tumor line was dissociated after transplant generation one and plated onto T-75 flasks to develop a tissue culture line (49). The Met-1 cell line was cultured in high glucose DMEM supplemented by 10% fetal bovine serum, 2mM glutamine, and antibiotics (Sigma, St Louis, MO). The Met-1 cell line is currently in passage 30.

Immuno-reagents: Monoclonal rat anti-CD44 antibody (Clone:020; Isotype: IgG_{2b}; obtained from CMB-TECH, Inc., Miami, FL.) used in this study recognizes a common determinant of the CD44 class of glycoproteins including CD44s and other variant isoforms (20,30) and is capable of precipitating all CD44 variants. For the preparation of polyclonal rabbit anti-CD44v3 or rabbit anti-MMP-9 [the whole molecule of MMP-9 or metal binding region peptides of 92 kDa], specific synthetic peptides [\approx 15-17 amino acids unique for either CD44v3 sequences or MMP-9 sequences, respectively] were prepared, respectively by the Peptide Laboratories of Department of Biochemistry and Molecular Biology using an Advanced Chemtech

automatic synthesizer (model ACT350). Conjugated CD44v3 or MMP-9 peptides (to hemocyanin or polylysine) were injected into rabbits to raise the antibodies. The anti-CD44v3 or anti-MMP-9 sera were collected from each bleed and stored at 4°C containing 0.1% azide. Both rabbit anti-CD44v3 and rabbit anti-MMP-9 IgGs were prepared using conventional DEAE-cellulose chromatography (9) and were tested to be monospecific (by immunoblot assays).

Metabolic Labeling and Enzymatic Digestion:

Confluent Met-1 cells ($\approx 1 \times 10^6$ /60 mm dish) were incubated with sulfate free Joklik medium for 6 h followed by ($^{35}\text{SO}_4$)²⁻ labeling (300 $\mu\text{Ci/ml}$) for 4h at 37°C. These radioactive sulfate-labeled cells were washed in phosphate buffered saline pH 7.2 (PBS) and solubilized in RIPA buffer (28-30). Subsequently, these ^{35}S -labeled cells were used for anti-CD44-mediated immunoprecipitation as described below.

Cell Surface Labeling Procedures:

Met-1 cells suspended in PBS were surface labeled using the following biotinylation procedure. Briefly, cells (10^7 cells/ml) were incubated with sulfosuccinimidobiotin (Pierce Co., Rockford, IL) (0.1mg/ml) in labeling buffer (150 μM NaCl, 0.1M HEPES, pH 8.0) for 30 min at room temperature. Cells were then washed with PBS to remove free biotin. Subsequently, the biotinylated cells were used for anti-CD44-mediated immunoprecipitation as described below.

Immunoprecipitation and Immunoblotting Techniques:

Unlabeled or labeled (surface biotinylated or radioactive sulfate labeled) cells were washed in 0.1M phosphate buffered saline (PBS; pH 7.2) and solubilized in RIPA buffer (28,30). The solubilized extracts were incubated with rat anti-CD44 antibody at 4°C for 15 h followed by incubation with goat anti-rat IgG agarose beads at 4°C for 90 min as described before (28-30). The immunoprecipitates were analyzed by 7.5% SDS polyacrylamide gel electrophoresis followed by fluorography. In some cases, Met-1 cells were solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 buffer and immunoprecipitated

using rat anti-CD44 antibody followed by goat anti-rat IgG. The immunoprecipitated material was solubilized in SDS, electrophoresed and blotted onto the nitrocellulose. After blocking non-specific sites with 3% bovine serum albumin, the nitrocellulose filter was incubated with rabbit anti-CD44v3 antibody (5µg/ml) or rabbit anti-MMP-9 antibody (5µg/ml) at room temperature followed by incubation with horse radish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution) at room temperature for 1 h. The blots were developed using Renaissance chemiluminescence reagent (NEN, DuPont, Boston, MA) according to the manufacturers instructions.

ELISA Assays:

Purified CD44v_{3,8-10} was obtained by solubilizing unlabeled Met-1 cells with non-ionic detergent Triton X-100 followed by rabbit anti-CD44v3 immunoaffinity chromatography. Various concentrations of purified CD44v_{3,8-10} were digested with 1mU of Flavobacterium heparinum Heparitinase and/or 25mU Proteus Vulgaris Chondroitin ABC lyase (ICN Immunobiologicals, Lisle, IL) for 1h at 37°C. Both untreated and enzyme-treated CD44v_{3,8-10} were immobilized to Immulon 2 plates (Dynatech Laboratories, Chantilly, VA) in PBS overnight. After the plates were blocked and washed with 0.05% Tween-20/PBS, 0.5µg/ml of biotinylated basic fibroblast growth factor (b-FGF) or biotinylated vascular endothelial growth factor (VEGF) (R & D System Inc., Minneapolis, MN) was added to the wells in the presence and absence of 3mg/ml heparin (Sigma Co.,) and incubated for 1h at room temperature. The plates were washed and avidin-conjugated HRP (Sigma Co.) was added to the wells for 30min at room temperature. These plates were then re-washed and incubated with 100µl of EIA chromogen Reagent for 10min at room temperature. The reaction was terminated by adding 100µl of 1N H₂SO₄, and the optical density measured on an ELISA reader at two wavelengths 450 and 630nm as described previously (2).

Cell Adhesion Assay:

Met-1 cells were metabolically labeled with Tran³⁵S-label (20 μ Ci/ml) as described above. After labeling, the cells were washed in PBS and incubated in PBS containing 5mM EDTA at 37°C to obtain a non-adherent single cell suspension. Labeled cells ($\approx 9.1 \times 10^5$ cpm/ 10^5 cells) were incubated on HA-coated plates (prepared as described previously) (5,30) at 4°C for 30 min either alone or in the presence of a unique monoclonal rat anti-CD44 antibody [IRAWB-14] (50 μ g/ml) (a gift from Dr. Robert Hyman, Salk Institute, CA). Following incubation, the wells were washed three times in PBS, the adherent cells were solubilized in PBS containing 1% SDS, and the well-bound radioactivity was determined by liquid scintillation counting.

Double Immunofluorescence Staining:

Met-1 cells were incubated with rat anti-CD44 antibody (clone:020) (10 μ g/ml) at room temperature for 30 min followed by incubation with fluorescein-labeled goat anti-rat antibody (10 μ g/ml) for 15min at room temperature to induce "invadopodia" formation (34,35). In some cases, cells were pre-treated with various inhibitors [e.g. cytochalasin D (20 μ g/ml), W-7 (20 μ M) or colchicine (1×10^{-5} M) at room temperature for 30 min followed by incubating with rat anti-CD44 antibody (10 μ g/ml) and fluorescein-labeled goat anti-rat antibody (10 μ g/ml) for 15min at room temperature to test the effects of these drugs on "invadopodia" formation. Subsequently, cells were washed with PBS [0.1M phosphate buffer (pH 7.5) and 150mM NaCl] buffer and fixed by 2% paraformaldehyde. These fluorescein-labeled cells were then rendered permeable by methanol treatment and stained with monoclonal mouse anti-ankyrin (ANK016) antibody (5) or rabbit anti-MMP-9 antibody followed by rhodamine-conjugated goat anti-mouse IgG or rhodamine-conjugated goat anti-rabbit IgG. To detect non-specific antibody binding, fluorescein-labeled cells were incubated with normal mouse or rabbit serum followed by rhodamine-conjugated goat anti-mouse IgG or rhodamine-conjugated goat anti-rabbit IgG. No labeling was observed in such control samples.

The fluorescein- and rhodamine-labeled samples were examined with a confocal laser scanning microscope (MultiProbe 2001 Inverted CLSM system, Molecular Dynamics, Sunnyvale, CA).

Binding of 125 I-labeled CD44v_{3,8-10} To Ankyrin:

Purified 125 I-labeled CD44v_{3,8-10} (≈ 0.32 nM protein, 1.5×10^4 cpm/ng) was incubated with 30 μ l of ankyrin conjugated to sepharose beads (≈ 0.75 μ g protein) in 0.5 ml of the binding buffer (described above). Binding was carried out in the presence or absence of various concentrations (1 nM - 1 μ M) of unlabeled competing synthetic peptide (NGGNGTVEDRKPSSEL-corresponding to the ankyrin binding sequence of CD44) (31) at 4°C for 5 h under equilibrium conditions. Equilibrium conditions were determined by performing a time course (e.g. 1 h-10 h) of the binding reaction. Following binding, the beads were washed in the binding buffer and the bead bound radioactivity was determined. Non-specific binding was determined in the presence of either a 100 fold excess of unlabeled ankyrin or using bovine serum albumin conjugated sepharose beads. Non-specific binding was approximately 20-30% of the total binding, and was subtracted from the total binding.

Reverse Transcriptase-Polymerase Chain Reaction and Southern Blot Analysis:

Total RNA was extracted from Met-1 cells by using the method of Chomczynski and Sacchi (11). Approximately 3 μ g of total RNA was used to synthesize first strand oligo (dT)-primed cDNA at 42°C for 1 h by a reverse transcription system (Promega, Madison, WI) containing avian myeloblastosis virus reverse transcriptase. Following first strand synthesis, PCR amplification of cDNA was carried out with an initial melting of the RNA/cDNA hybrid at 94°C for 30s, annealing at 60°C for 30s, and polymerization at 72°C for 1 min. One pair of PCR primers was utilized to amplify between exon 5 and v3 to detect any splice variants of CD44 mRNAs containing v1, v2, v3. Another pair was designed to amplify between v3 and ex15 to detect CD44 mRNAs containing v3-v10. The PCR primers used in this study were as follows: CD44 ex5 left primer (5'-

ACATCAGTCACAGACCTGCCC-3'); CD44 ex15 right primer (5'-ATCCATGAGTCACAGTGCGG-3'); CD44v3 right primer (5'-CTGAGGTGTCTGTCTCTTTC-3'); CD44v3 left primer (5'-GACTCCACAGACAGAGAAAG-3'). A negative control was carried out in the absence of reverse transcriptase, and a positive control was carried out using human CD44v (v3,v8,v9,v10) cDNA as a template. The PCR products were examined on 2.0% agarose gel followed by Southern blot analysis hybridizing with human CD44 cDNA probes using the protocol previously described (20). The PCR products were also one-step cloned using TA-cloning kit (Invitrogen, San Diego, CA) and sequenced by dideoxy sequencing method (20).

Zymography and Immunoprecipitation:

Gelatin zymography utilized a modified procedure of Herron et al. (16,19) for detecting picograms of MMP-2, MMP-9 and nanograms of other MMPs and proteases. SDS-PAGE was performed in 7.5% or 10% polyacrylamide containing 0.33 mg/ml gelatin. The gels were then rinsed twice in 0.25% Triton X-100, and incubated in the assay buffer [0.05 M Tris-HCl (pH 7.5), 0.2 M NaCl, 0.01 M CaCl₂, 1 μ M ZnCl₂, 3 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN₃ and 0.005% Brij 35] at 37°C for 18h. Gels were then stained with Coomassie blue R 250. Both latent and active forms of gelatinases or other MMPs produce clear areas in the gel. Each gel lane contains pre-estimated amount of MMPs.

Immunoprecipitation analysis was performed according to standard methods (18). Serum-free media, Met-1 membrane fractions (28-30) or anti-CD44-associated immune complexes were collected and analyzed for gelatinases or other MMP(s) content by zymography or substrate assays. In addition, conditioned serum-free media or Met-1 membrane fractions containing MMP(s) were immunoprecipitated with monospecific rabbit anti-MMP-9 IgG(s) using protein A-agarose suspensions (50-95 μ g of protein A) at 4°C. A negative control, normal rabbit IgG or preimmune serum [in which endogenous MMPs (specially gelatinases) were removed by gelatin-sepharose affinity columns] was used. After the reacted agarose gels were washed three times with the buffer [0.05M Tris-HCl (pH 7.5), 0.2M NaCl, 0.01M CaCl₂, 1 μ M

ZnCl₂, 3mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN₃ and 0.005% Brij 35], the immune complexes were then analyzed by zymography for specific enzyme activity or for proteins by SDS-PAGE.

The Met-1 membrane fraction or conditioned serum-free media was also used to quantitate for the MMP-2 or MMP-9 gelatinase (MMPs) activity using ³H-acetylated Type I gelatin (16,40). Assays were set up in the presence or absence of 1.0 mmol/L aminophenylmercuric acetate (APMA) to measure latent and active enzymes; Negative control samples containing 2 mmol/L 1, 10-phenanthroline were also run in the assays.

RESULTS

Expression of CD44 Variant Isoform(s) in Met-1 Cells:

The expression of CD44 variant isoforms is known to be closely correlated with metastatic and proliferative behavior of a variety of tumor cells including various carcinomas such as human breast tumor cells (6,12). To directly examine CD44 isoform expression on the surface of Met-1 cells, we have utilized surface biotinylation techniques and a specific monoclonal anti-CD44 antibody that recognizes the CD44 epitope located at the N-terminus of the common domain (20,30). Our results indicate that a single surface-biotinylated polypeptide (M. W. \approx 260kDa) displaying immunological cross-reactivity with CD44 is preferentially expressed on the surface of Met-1 (Fig. 1A). This surface labeled 260Da protein can also be immunoprecipitated (Fig. 1B) or immunoblotted (Fig. 2D) by rabbit anti-CD44v₃ antibody raised specifically against the v₃ sequence suggesting that this protein is a CD44v₃-containing isoform. No CD44v₃-containing material is observed in control samples when either normal rat IgG or preimmune rabbit serum is used (Fig. 2F).

To verify the expression of CD44v₃-containing transcript(s) at the RNA level, total RNA from Met-1 cells was extracted and analyzed by RT-PCR using exon specific primers. Using a PCR primer pair to amplify between v₃ and exon 15 by RT-PCR (Fig. 2C-a), a single product is detected at approximately 530 bp as shown

in Fig. 2A (lane 1). The size of the amplified fragment appears to correspond to a known CD44 variant isoform which contains v3 and v8-10 exon insertions. Using a PCR primer pair to amplify between exon 5 and v3 (Fig. 2C-b), one amplified DNA fragment is observed [about 270 bp as shown in Fig. 2B (lane 1)] that is identical to an amplicon from a human variant CD44 cDNA template containing v3, v8-v10 exons (Fig. 2B, lane 2). The RT-PCR reaction is specific since no amplified fragment can be detected in samples without any reverse transcriptase (Fig. 2A, lane 2; and 2B, lane 3). This V3-containing PCR product was subsequently "one-step cloned" into the pCRTM vector from Invitrogen Corp. and sequenced. Our nucleotide sequence data indicate that only v3 (but not v1 nor v2) is present in CD44 transcripts expressed in Met-1 cells and that the variant exon structure of CD44 transcripts containing v3 in Met-1 cells is the CD44v_{3,8-10} isoform as shown in Fig. 2C-c. This CD44v_{3,8-10} variant exon structure was previously identified in human breast carcinoma samples (6,20) and its molecular mass (expressed at the protein level) has been shown to be ~260kDa (2). Therefore, we believe that the major CD44v₃-containing isoform expressed on the surface of Met-1 cells is the CD44v_{3,8-10} isoform.

Hyaluronic Acid (HA)-Mediated Cell Adhesion By Met-1 Cells:

CD44 is the major hyaluronan cell surface receptor (46), and a cellular adhesion molecule in many different cell types (3,26). Specific hyaluronic acid (HA) binding motifs have been identified and localized in the extracellular domain of a number of CD44 isoforms (48). In this study we have examined the ability of Met-1 cells to adhere to HA-coated plates. Our data indicate that Met-1 cells (without any treatment) display a relatively low level of CD44-specific cell adhesion to HA-coated plates (Fig. 3A). After Met-1 cells were treated with specific rat anti-CD44 antibody (IRAWB 14) known to upregulate CD44 expression on the cell surface (25), the cells display approximately a 5-fold increase of HA-mediated cell adhesion (Fig. 3B). These findings suggest that the CD44v_{3,8-10}

isoform contains a CD44-specific hyaluronan receptor required for HA-mediated cell adhesion in Met-1 cells.

Attachment of Vascular Endothelial Growth Factor (VEGF) To

CD44v_{3,8-10} in Met-1 Cells:

A number of CD44 isoforms, including CD44v_{3,8-10}, have been shown to contain sulfated oligosaccharides (2,30). Consequently, we have metabolically labeled Met-1 cells with ($^{35}\text{SO}_4$)²⁻ and then looked for the presence of radioactive sulfate label in CD44v_{3,8-10} using anti-CD44v3-mediated immunoprecipitation (Fig. 1C). Our results clearly indicate that $^{35}\text{SO}_4^{2-}$ is incorporated into the glycosaminoglycan (GAG) chains of CD44v_{3,8-10} (Fig. 1C). Previously, it has been suggested that the GAG chains of certain CD44v3-containing isoform are involved in the binding of heparin binding growth factors such as basic fibroblast growth factor (bFGF) (2). In this study we tested the possible interaction between CD44v_{3,8-10} and two heparin binding growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Our results clearly indicate that CD44v_{3,8-10} binds preferentially to VEGF (Fig. 4A-a; and 4B-a), but not bFGF (Fig. 4A-b). This binding between CD44v_{3,8-10} and VEGF is reduced in the presence of heparin (Fig. 4A-c); and appears to be sensitive to the digestion by heparitinase (Fig. 4B-b) and/or chondroitinase (Fig. 4B-c and 4B-d), suggesting the GAG chains of CD44v_{3,8-10} are responsible for VEGF binding.

Transmembrane Interaction Between CD44v_{3,8-10} and Ankyrin:

In this study we have employed a monoclonal anti-CD44 antibody to induce membrane spikes or "invadopodia" formation on Met-1 cells (Fig. 5). Interestingly, surface CD44v_{3,8-10} appears to be preferentially accumulated as a receptor capping-like structure at the leading edge of the "invadopodia" structure. This anti-CD44-induced "invadopodia" formation and CD44v_{3,8-10} accumulation are readily inhibited by cytochalasin D (a microfilament inhibitor) and W-7 (a calmodulin antagonist) but not colchicine (a microtubule inhibitor)

(Table 1). These findings indicate that microfilament-associated components are involved in these events.

Double labeling and confocal microscopic analysis shows that CD44v_{3,8-10} is preferentially localized with certain cytoskeletal proteins, such as ankyrin, in these "invadopodia" structures (Fig. 5A). Furthermore, we have used competition binding assays and a synthetic peptide identical to the ankyrin binding domain (e.g. NGGNGTVEDRKPSEL) to analyze the CD44v_{3,8-10}'s ankyrin binding. Our results indicate that the synthetic peptide corresponding to the CD44 isoform's ankyrin binding domain competes effectively with

CD44v_{3,8-10} in binding to ankyrin with an apparent inhibition constant (K_i) \approx 0.7nM (Fig. 6). These findings strongly suggest that an interaction between CD44v_{3,8-10} and the cytoskeleton occurs at the cytoplasmic region of CD44v_{3,8-10} (i.e. ³⁰⁶NGGNGTVEDRKPSE³²⁰L) and is critically important for the activation of "invadopodia" formation.

Association Of CD44v_{3,8-10} Isoform and Matrix Metalloproteinases, MMP-9 in Met-1 Cells

Metastatic tumor cells are capable of degrading the extracellular matrix (ECM) barrier in order to migrate out of the primary tumor location and into the circulation in order to establish a site of metastasis (7,33). The breakdown of the ECM can be traced to the action of one or more matrix metalloproteinases (MMPs) which belong to a family of zinc proteases (32,36,47). Normally, they are secreted as proenzymes and become activated outside the cell by serine proteases, such as plasmin, by the removal of a 9kDa prosegment from the active site. Several MMPs, such as the 72kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), the 92kDa gelatinase (gelatinase B, type V collagenase, MMP-9), the 57kDa stromelysin (MMP-3) and interstitial collagenase (MMP-1), are likely to be responsible for ECM degradation during tumor invasion and metastasis (7,43).

In this study we have employed a number of techniques, including zymographic assays, immunoprecipitation techniques and immunofluorescence staining to examine

the relationship between gelatinase activities and the CD44v_{3,8-10} isoform in Met-1 tumor cells. The results of the gelatin zymographic analyses indicate that the lytic band of mouse gelatinase in the membrane fraction of Met-1 cells is approximately 92kDa (Fig. 7A-a). No other lytic bands of gelatinase are observed. Using anti-human MMP-9-mediated immunoprecipitation of Triton X-100 solubilized Met-1 membranes, we have found that this 92kDa lytic gelatinase band detected in Met-1 membranes (Fig. 7A-a) corresponds to the lytic band of human MMP-9 (Fig. 7A-b). As a control, the zymographic profile of anti-MMP-9 IgG alone (without using Triton X-100 solubilized Met-1 membranes and immunoprecipitation) shows no lytic bands of gelatinase (Fig. 7A-d). This result suggests that the lytic band of 92kDa gelatinase detected in the membrane fraction of Met-1 cells (Fig. 7A-b) corresponds to a MMP-9-like molecule.

It has been determined previously that the lytic band corresponding to the latent form of normal mouse MMP-9 is approximately 105kDa (38,44) (Fig. 7B-a). Our data indicate that the 92kDa gelatinase from Met-1 membrane fraction appears to be slightly smaller than normal latent form of mouse MMP-9 (105kDa) (Fig. 7B-b). The reduced molecular weight of Met-1's MMP-9 suggests that this gelatinase is in an active form. Treatment of the Met-1 membrane fraction with alpha(2)-macroglobulin (an inhibitor known to bind active metalloprotease activity) (37) results in blockage of the lytic reaction of 92kDa gelatinase activity (Fig. 7B-c). Measurement of the gelatinase activity using ³H-acetylated Type I gelatin (16,40) indicates that the MMP-9 associated with Met-1 membranes is an active gelatinase. This enzyme does not require aminophenylmercuric acetate (APMA) to become active; and its activity can be readily inhibited by 1, 10-phenanthroline (data not shown). These findings further support the notion that the lytic band of the 92kDa (mouse MMP-9) associated with Met-1 membranes is present in a proteolytically active form. In addition, we have observed a broad lytic band (possibly a secreted form of MMP-9) in the serum free conditioned media from Met-

1 cells which may represent the lytic bands of both the latent (105kDa) and active (92kDa) mouse gelatinase (Fig. 7A-f).

Further analyses by double immunofluorescence labeling and confocal microscopy indicate that the 92kDa (mouse MMP-9-like molecule) is co-localized with CD44v_{3,8-10} in the "invadopodia" structure of the Met-1 cells (Fig. 5B). In order to explore the possible physical association between these two molecules, we have carried out anti-human MMP-9-mediated immunoprecipitation of Triton X-100 solubilized Met-1 membranes followed by immunoblotting with anti-CD44v₃ antibody. Our data show that CD44v_{3,8-10} is co-precipitated with the metalloprotease (e.g. MMP-9) as a complex (Fig. 1E). In addition, we have carried out anti-CD44-mediated precipitation followed by gelatin zymography analysis. Our results indicate that the 92kDa lytic gelatinase band can be detected in the anti-CD44-immunoprecipitated materials (Fig. 7A-c), suggesting these two molecules are tightly associated with each other.

DISCUSSION

A number of studies indicate that the expression of CD44 on the cell surface changes profoundly during tumor metastasis, particularly during the progression of various carcinomas (1,6,12). Many tumor cells and tissues have been found to express different CD44 variant (CD44v) isoforms (1,6,12). These isoforms, containing additional exon insertions (e.g. exons 6-11) within the CD44 membrane proximal region appear to be associated with tumor metastasis (1,6,12). In fact, CD44 variant expression has been used as an indicator of metastasis. In this paper we have chosen Met-1 cells, which were derived from high metastatic potential tumors, as a model system to analyze the relationship between CD44 isoform expression and breast tumor progression. Using a variety of techniques, including RT-PCR, Southern blot and immuno-blot, we have shown that Met-1 cells express a single CD44 isoform (M.W. \approx 260 kDa) which contains a v_{3,8-10} exon insertion. This CD44v_{3,8-10} isoform has been found to be closely associated with tumor metastasis during human breast cancer progression. Since information

regarding the role of CD44v_{3,8-10} in regulating mammary tumor cell function is very limited at the present time, it is clearly important to examine the structural properties of this molecule.

Previously, CD44v_{3,8-10} [with heparan sulfate (GAG) addition] was found to bind bFGF on COS cells (2). In this study we have found that the external domain of sulfated CD44v_{3,8-10} (Fig. 2C) in Met-1 cells contains heparan sulfate (GAG) addition sites which preferentially bind VEGF, but not bFGF (Fig. 4). It is possible that a different organizational and/or conformational presentation of CD44v_{3,8-10} molecules on the surfaces of Met-1 cells and COS cells may result in the selective binding of certain growth factors to these two types of cells. The fact that VEGF binds CD44v_{3,8-10} on the surface of Met-1 cells suggests that CD44v_{3,8-10} may serve as a modified VEGF receptor on the tumor cell surface. The effects of VEGF on CD44v_{3,8-10}-mediated Met-1 cell activation are currently under investigation. VEGF is a specific mitogen for endothelial cells and a potent microvascular permeability factor (14,15). It plays an integral role in angiogenesis and thus in potentiation of solid tumor growth (14,15). Preliminary data indicate that Met-1 cells are capable of inducing a high level of intratumoral microvessel formation (49). Therefore, the attachment of VEGF to the heparin sulfate sites on CD44v_{3,8-10} may be responsible for the onset of breast tumor-associated angiogenesis.

The binding of HA to CD44 is conferred by two regions in the N-terminal extracellular domain (48). HA binding can be induced by certain activating antibodies such as rat anti-CD44 antibody (IRAWB 14) (25). CD44v isoforms all contain similar HA binding motifs (48), certain CD44v isoforms have been reported to display significantly less HA binding than CD44s (13,42). Our data indicate that Met-1 cells, expressing the CD44v_{3,8-10} isoform, display a low level of cell adhesion to HA-coated plates (Fig. 3A). It is possible that post-translational modifications of the v_{3,8-10}-encoded structure induce surface rearrangements or conformational changes in the HA-binding domains which result in a reduction of

HA-mediated cell adhesion. The fact that rat anti-CD44 (IRAWB-14) is capable of promoting HA-mediated cell adhesion suggests that the HA binding motifs in the CD44v_{3,8-10} isoform may be readily regulated (either up or down) during the onset of tumor cell transformation leading to increased cell motility (migration) and invasion.

The invasive phenotype of tumor cells characterized by tumor cell motility (22,24) and "invadopodia" formation (34,35), is clearly linked to cytoskeletal function. Dissection of the transmembrane pathways controlling these cellular processes should aid in understanding the regulatory mechanisms underlying tumor invasion and metastasis. Previously, we have demonstrated that CD44 isoforms [e.g. CD44s and CD44 variant (ex14/v10)] display certain high affinity binding to the cytoskeletal protein, ankyrin (3-6;28-31). The cytoplasmic domain of the CD44 isoforms contain the ankyrin binding site (NGGNGTVEDRKPSSEL) which is $\geq 90\%$ conserved in all of the CD44 isoforms (31) and resides between amino acids 305 and 320 of the CD44 molecule. This ankyrin binding sequence appears to be required for regulating CD44-mediated function (3,5,6). In this paper, using double labeling and confocal microscopic analyses, we have shown that CD44v_{3,8-10} is closely associated with ankyrin in the "invadopodia" structures (Fig. 5). The fact that the synthetic peptide (NGGNGTVEDRKPSSEL)--corresponding to CD44 isoform's ankyrin binding domain competes effectively with CD44v_{3,8-10} to bind ankyrin ($K_i \approx 0.7\text{nM}$) (Fig. 6) suggests that the sequence of "NGGNGTVEDRKPSSEL" in the cytoplasmic domain of CD44v_{3,8-10} interacts directly with ankyrin. These results strongly suggest that the cytoskeletal protein, ankyrin, plays an important role in regulating CD44v_{3,8-10}-associated "invadopodia" formation needed for cell motility and invasion.

It has been suggested that a number of different matrix metalloproteinases (MMPs) [including the 72kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), the 92kDa gelatinase (gelatinase B, type V collagenase, MMP-9), the stromelysins (MMP-3, MMP-11) and the interstitial fibroblast-type collagenase

(MMP-1)] are thought to play an important role in degrading extracellular matrix materials (ECM) during tumor invasion and metastases (7,10,33). Biochemical interactions between MMPs and various cell surface molecules have not been fully established. A membrane-type matrix metalloproteinase (MT-MMP, a transmembrane MMP) together with TIMP-2, a tissue inhibitor of metalloproteinase-2 (MMP-2), have been reported to be involved in the activation of MMP-2 on the cell surface (39). Recently, Brooks et al 1996 (8) determined that matrix metalloproteinase MMP-2 is localized in a proteolytically active form on the surface of invasive cells based on its ability to bind directly to integrin $\alpha v \beta 3$. The question of whether other MMPs are also involved in interacting with CD44-related adhesion molecules during the progression and metastasis of tumors remains to be answered.

In this study we have found that CD44_{v3,8-10} (a surface adhesion molecule) is closely associated with MMP-9 (gelatinase B) in the plasma membrane of Met-1 cells based on the evidence provided by anti-MMP-9 and anti-CD44-mediated immunoprecipitation followed by immunoblot and gelatin zymography (Figs. 1E; and 7A-c). Furthermore, CD44V_{3,8-10}-associated MMP-9 appears to be present in a proteolytically active form (Fig. 7B-b,c) and preferentially localized at the "invadopodia" structure of the Met-1 cells (Fig. 5). Our results are consistent with previous findings showing that certain protease(s) is(are) localized on "invadopodia" of human malignant melanoma cells (10). Therefore, we believe that the close interaction between CD44v_{3,8-10} and the active form of MMP-9 in the "invadopodia" structure of Met-1 tumor cells may be required for the degradation of extracellular matrix (ECM) for tumor cell invasion and metastasis. Preliminary data indicate that MMP-9 is complexed with the cytoplasmic domain (not the extracellular portion) of the CD44V_{3,8-10} molecule (data not shown). The nature of the linkage between these two molecules is currently undergoing investigation.

In conclusion, we propose that CD44v_{3,8-10} and associated molecules (e.g. VEGF and metalloprotease) play a pivotal role in regulating metastatic tumor cell

behavior (e.g. invadopodia formation and matrix degradation) and promoting angiogenesis during breast cancer progression.

(7) CONCLUSION

In this study we have employed a unique breast cancer cell line [Met-1 which was derived from a high metastatic potential tumor in transgenic mice expressing polyomavirus middle T oncogene] to study the role of CD44 variant isoform(s) in the regulation of metastatic breast tumor cell behavior. The results of RT-PCR, Southern blot, immunoprecipitation and immuno-blot analyses indicate that these cells express a major CD44 isoform (M.W. \approx 260 kDa) containing a v3,8-10 exon insertion (designated as CD44v_{3,8-10}). In addition, biochemical analyses indicate that the external domain of CD44v_{3,8-10}, which contains added heparan sulfate, preferentially binds vascular endothelial growth factor (VEGF), but not basic fibroblast growth factor (bFGF). These findings suggest that CD44v_{3,8-10} is involved in the onset of tumor-associated angiogenesis.

We have determined that untreated Met-1 cells display low level cell adhesion to hyaluronic acid (HA)-coated plates. Following the addition of anti-CD44 antibody, the Met-1 cells attach strongly to HA-coated plates and form membrane spikes or "invadopodia" structures via a microfilament-dependent process. Double labeling and confocal microscopic analysis show that CD44v_{3,8-10} is preferentially localized with certain cytoskeletal proteins, such as ankyrin, in the "invadopodia" structures. Further in vitro binding data support the notion that the cytoplasmic domain of CD44v_{3,8-10} interacts directly with ankyrin. Furthermore, using zymography assays and double immunofluorescence staining, we have found that CD44v_{3,8-10} is closely associated with the active form of matrix metalloproteinase, MMP-9 in a complex within the "invadopodia" structures. Therefore, we propose that CD44v_{3,8-10} and associated molecules (e.g. VEGF and MMP-9) play an essential role in regulating metastatic tumor cell behavior (including invadopodia formation, matrix degradation and angiogenesis) during the progression of breast cancer.

Future Work: In the coming year, we plan to address the following two specific aims:

Aim 1: To construct CD44v3 deletion or site-directed mutants lacking specific functional domains for cytoskeleton binding. CD44v3 mutant polypeptides will then be expressed in non-metastatic human breast epithelial cells and their structural changes will be correlated with tumor cell motility, invasiveness, and metastasis.

Aim 2: To examine human breast tissue samples for CD44v3 expression and correlate qualitative and quantitative levels with the invasive and/or metastatic phenotype during breast cancer progression. This will allow us to establish CD44v3 as a useful metastatic marker for breast cancer detection and prognosis.

Specifically, the following experiments will be carried out:

Aim 1: To construct CD44v3 deletion or site-directed mutants lacking specific functional domains for cytoskeleton binding. CD44v3 mutant polypeptides will then be expressed in nonmetastatic human breast epithelial cells and their structural changes will be correlated with tumor cell motility, invasiveness, and metastasis.

The procedures for cloning and expression of CD44v3 in human breast cancer cells are described as follows: The RT-PCR primers (i.e. TACATCAGTCACAGACCTGC and ATCCATGAGTGGTATGGGAC) will be used to permit the direct cloning of the CD44v3-related amplification products into pRc/CMV/CD44 for expression in mammalian cells. The amplification products will contain unique restriction enzyme sites for Hpa I (476) and Tth I(835). The Hpa I site is also unique in the human CD44s insert of pRc/CMV/CD44s (provided by Dr. Eugene Butcher, Stanford University), while there are two sites for Tth I. Therefore, the amplification products can be inserted directly into pRc/CMV/CD44s which has been partially digested with Tth I and completely digested with Hpa I. This will allow the insertion of the

CD44v3 sequences directly into the CD44s coding sequence of pRc/CMV/CD44s at precisely the correct location and reading frame. Final constructs containing full-length CD44v3 structures will be used for the transfection of eukaryotic cells. In addition, we plan to create deletion and/or site-directed mutants that lack particular sequences for ankyrin-binding in the cytoplasmic domain.

These CD44v3 constructs will then be transfected into a non-invasive human breast cancer cell lines such as MCF10A and HBL100 and characterized functionally by analyzing the effects on CD44v3-cytoskeleton interaction, invasion and metastasis. In vitro characterization of individual CD44v3 deletion mutants will focus on CD44v3-cytoskeleton interaction using the same procedures as described above. Using information obtained from mutational analysis, we will then correlate the expression of functional domains specific for CD44v3-cytoskeleton association with motility and invasive/metastatic behavior in breast cancer cells. Cellular motility will be performed using in vitro invasion assays and "invadopodia" formation analysis as described previously. Tumorigenesis and invasive/metastatic properties of individual transfectants will be evaluated by subcutaneous (or by mammary fat pad or tail vein) injection in 6-week old female nude mice with quantitation by the size and number of tumors formed as well as various sites of metastases as compared to nontransfected controls. Approximately, 7-10 mice per experimental group will be injected with 1×10^7 cells. After specific CD44v3 domains have been identified as critical to cytoskeleton binding and/or metastasis, we will further determine key amino acids by site-directed mutagenesis. These may involve conserved residues within known consensus sequences or previously unidentified sequences. Identification of these key regions will provide further clues to mechanistic aspects underlying breast cancer metastasis.

Anticipated Results: We predict that CD44v3's functional domains related to cytoskeletal binding site(s) will be mapped out using both deletion and site-directed mutagenesis methods.

Aim 2: To examine human breast tissue samples for CD44v3 expression and correlate qualitative and quantitative levels with the invasive and/or metastatic phenotype during breast cancer progression. This should allow us to establish CD44v3 as a useful metastatic marker for breast cancer detection and prognosis.

The procedures used for generating polyclonal and monoclonal antibodies against CD44v3 will be performed as described previously. Standard antibody characterization methods such as ELISA, immunoblotting, and immunoprecipitation will be used to verify the specificity and titers of polyclonal and monoclonal antibodies. Specifically, breast cancer tissue sections [e.g. node positive vs node negative breast cancer samples; and high- vs low-grade histologies of ductal carcinoma in situ (DCIS)] will be compared. In order to control for other prognostic factors, we will limit the analysis of invasive cancers to tumors of similar size (i.e. between 1 and 3 cm) and nuclear grade (low to intermediate). The samples will be fixed with 2% paraformaldehyde and processed for standard immunoperoxidase staining using polyclonal or monoclonal anti-CD44v3 antibody. Some samples will also be processed for immunofluorescence staining using a laser scanning confocal microscope (MultiProbe 2001 Invert CLSM System, Molecular Dynamics) for both qualitative and quantitative measurements. The relationship between CD44v3 expression and clinicopathological indices will be analyzed by Fisher's exact t test. Correlation between the fraction of CD44v3 positive tumor cells in normal, non-metastatic and metastatic breast tissues will be assessed by the Spearman rank correlation coefficient as described previously. The results of these experiments will allow us to establish a possible relationship between CD44v3 expression and the progression of human breast cancers.

Anticipated Results:

We anticipate that there will be a strong correlation between the level of CD44v3 expression and invasive/metastatic behavior. Moreover, domains previously identified as critical to cytoskeletal interaction and/or those associated with invasion and metastasis will be found to be upregulated in those breast cancers which involve axillary lymph nodes. In addition, we anticipate a correlation between expression of CD44v3 and DCIS lesion of high histologic grade (i.e., those expected to become invasive).

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(9) APPENDICES

Table 1: Effects of Various Drugs On "Invadopodia" Formation.

Treatments ^a	"Invadopodia" Formation (% of Total Cells) ^b
Control (No Treatment)	10
Anti-CD44 Antibody	50
Cytochalasin D + Anti-CD44 Antibody	<5
W-7 + Anti-CD44 Antibody	<5
Colchicine + Anti-CD44 Antibody	48

a: The concentrations of various reagents used in this experiment were: rat anti-CD44 (50µg/ml); cytochalasin D (20 µg/ml); W-7 (20 µM); colchicine (1×10^{-5} M).

b: The values expressed in this table for "invadopodia" formation represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than ±5%.

FIGURE LEGENDS

Fig. 1: Characterization of CD44 Expression in Met-1 cells.

- A: Immunoprecipitation of surface biotinylated Met-1 cells using monoclonal anti-CD44 antibody (recognizing CD44 epitope located at the N-terminus of the common domain).
- B: Immunoprecipitation of surface biotinylated Met-1 cells with rabbit anti-CD44v₃-specific antibody.
- C: Immunoprecipitation of Met-1 cells [metabolically labeled with (³⁵SO₄)²⁻] with rabbit anti-CD44v₃-specific antibody.
- D: Immunoblot of Met-1 cells with rabbit anti-CD44v₃-specific antibody.
- E: Co-immunoprecipitation of MMP-9 with CD44: Immunoprecipitation of Met-1 cells with rabbit anti-MMP-9 followed by immunoblot with rat anti-CD44 (recognizing CD44 epitope located at the N-terminus of the common domain).
- F: Immunoprecipitation of surface biotinylated Met-1 cells with normal pre-immune rabbit serum [or normal rat IgG (data not shown)].

Fig. 2: Reverse Transcriptase-Polymerase Chain Reaction and Southern Blot Analysis of CD44 Transcript Expression in Met-1 Cells.

Total RNA isolated from Met-1 cells were reverse-transcribed and subjected to PCR using PCR primer pairs as described below in C-a and C-b. Subsequently, RT-PCR products were analyzed by Southern blot hybridization as described in the Materials and Methods.

- A: A single RT-PCR product (~530bp) generated by CD44 v3 and exon 15 primer pairs (the primer design is shown in C-a) (lane 1). As a negative control, RT-PCR was carried out in the absence of reverse transcriptase (lane 2).

B: A single RT-PCR product (~270bp) generated by CD44 exon 5 and v3 primer pairs (the primer design is shown in C-b) (lane 1). As a positive control, a known CD44 variant isoform which contains v3 and v8-10 exon insertions was used (lane 2). As a negative control, RT-PCR was carried out in the absence of reverse transcriptase (lane 3).

C: Schematic Exon Map of CD44 in Mouse.

C-a: Specific CD44 v3 and exon 15 primer pairs used in Fig. 2A.

C-b: Specific CD44 exon 5 and v3 primer pairs used in Fig. 2B.

C-c: Schematic illustration of the CD44v_{3,8-10} isoform identified in Met-1 cells.

Fig. 3: Effects of Monoclonal Rat Anti-CD44 Antibody (IRAWB-14) On Cell Adhesion to HA-Coated Plates.

Met-1 cells labeled with Tran³⁵S-label (9.1×10^5 cpm/ 10^5 cells) were either untreated (as a control) (A) or pretreated with rat anti-CD44 IgG (IRAWB-14) (50 µg/ml) (B). Following treatment, the cells were incubated with HA-coated plates at 4° C for 30 min. The non-specific binding of cells to HA-coated plates was determined in presence of soluble HA and was subtracted. Each experiment was performed in duplicate and the results represent an average of three separate experiments with a standard deviation <±5%.

Fig. 4: Binding of VEGF and b-FGF to CD44v_{3,8-10} by ELISA.

A: VEGF binding to purified CD44v_{3,8-10} [without any enzymatic digestion] in the absence (4A-a) and presence of 3mg/ml heparin (4A-c); b-FGF binding to purified CD44v_{3,8-10} (4A-b).

B-a: VEGF binding to purified CD44v_{3,8-10} [without any enzymatic digestion].

B-b: VEGF binding to purified CD44v_{3,8-10} [digested with heparitinase].

B-c: VEGF binding to purified CD44v_{3,8-10} [digested with chondroitinase].

B-d: VEGF binding to purified CD44v_{3,8-10} [digested with heparitinase and chondroitinase].

[O.D.450/630 units represent the relative amounts of VEGF or bFGF binding to a constant level of purified CD44v_{3,8-10}]

Fig. 5: Double Immunofluorescence Staining and Confocal Analysis of "Invadopodia" Structures (indicated by arrow head) in Met-1 Cells.

A: Co-localization of surface CD44v_{3,8-10} and intracellular ankyrin using rat anti-CD44 antibody and mouse anti-ankyrin antibody followed by fluorescence-labeled goat anti-rat IgG and rhodamine-labeled goat anti-mouse IgG, respectively.

B: Co-localization of surface CD44v_{3,8-10} and intracellular MMP-9 using rat anti-CD44 antibody and rabbit anti-MMP-9 antibody followed by fluorescence-labeled goat anti-rat IgG and rhodamine-labeled goat anti-rabbit IgG, respectively.

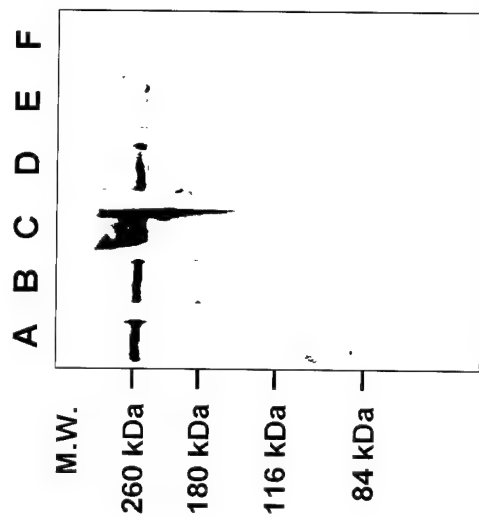
Fig. 6: Binding of ¹²⁵I-labeled CD44v_{3,8-10} To Ankyrin.

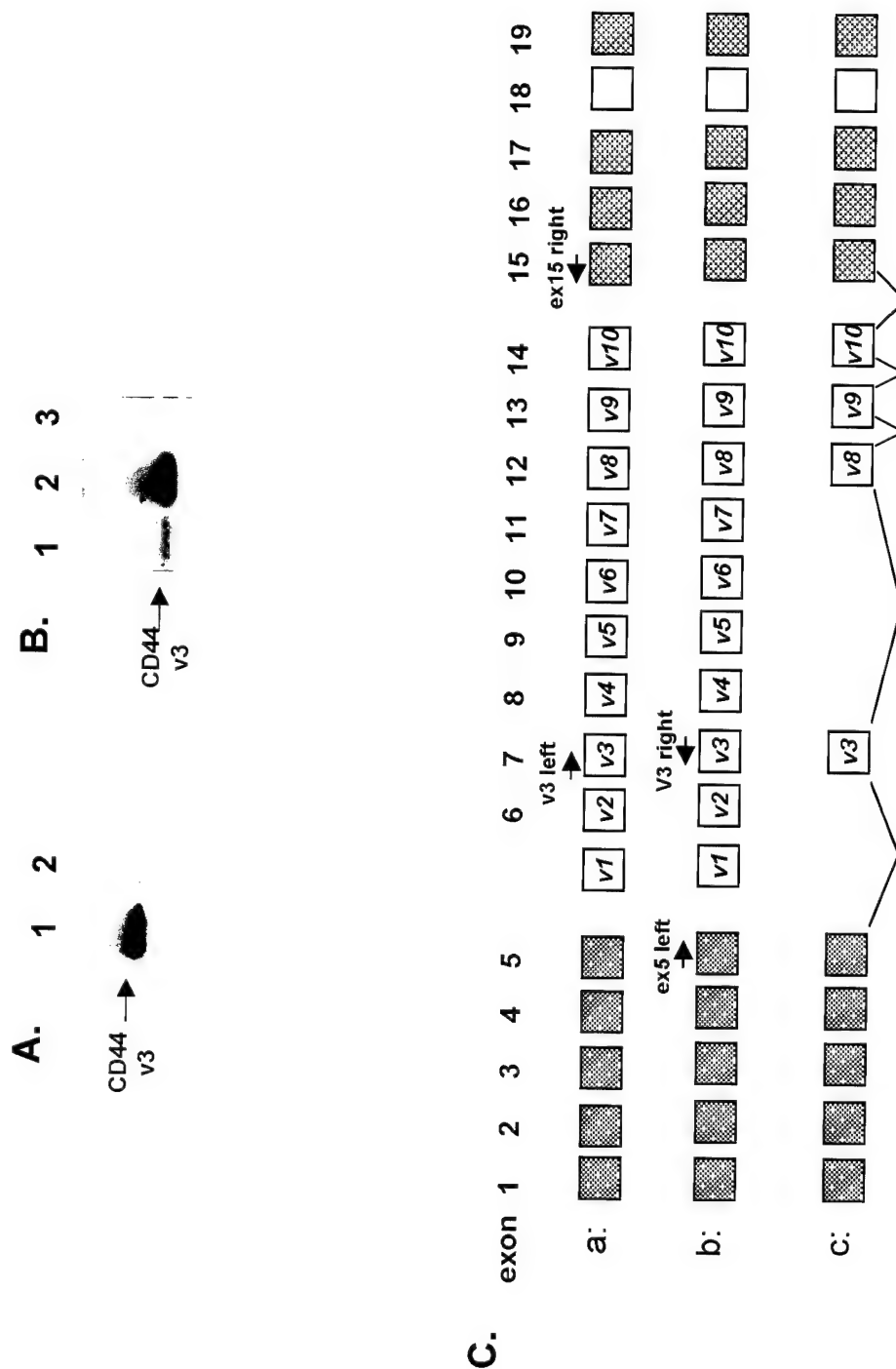
¹²⁵I-labeled CD44v_{3,8-10} was incubated with ankyrin in the presence of various concentrations of unlabeled synthetic peptide (NGGNGTVEDRKPSSEL) corresponding to the ankyrin-binding domain of CD44 as described in the Materials and Methods. The specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used.

Fig. 7: Gelatin Zymographic Analysis of MMPs in Met-1 Cells.

Gelatin zymography was used to detect MMPs in Met-1 cells according to the procedures described in the Materials and Methods.

- A-a: The 92kDa lytic gelatinase band detected in Met-1 membranes.
- A-b: The human MMP-9 (92kDa lytic gelatinase band) detected by anti-human MMP-9-mediated immunoprecipitation of Triton X-100 solubilized Met-1 membranes.
- A-c: The 92kDa lytic gelatinase band associated with the anti-CD44-immunoprecipitated materials.
- A-d: As a control, anti-MMP-9 IgG was used (in the absence of Triton X-100 solubilized Met-1 membranes and immunoprecipitation).
- A-e: As a control, anti-CD44 IgG was used (in the absence of Triton X-100 solubilized Met-1 membranes and immunoprecipitation).
- A-f: The secreted form of MMP-9 [containing both latent (105kDa) and active (92kDa) mouse gelatinase] collected from the serum free conditioned media containing Met-1 cells).
- B-a: The lytic band (\approx 105kDa) corresponding to the latent form of normal mouse MMP-9.
- B-b: The 92kDa gelatinase from Met-1 membrane fraction.
- B-c: Treatment of the Met-1 membrane fraction with alpha(2)-macroglobulin (an inhibitor known to inactivate metalloprotease activity).
- B-d: Human MMP-9 (92kDa) and MMP-2 (72kDa) markers.





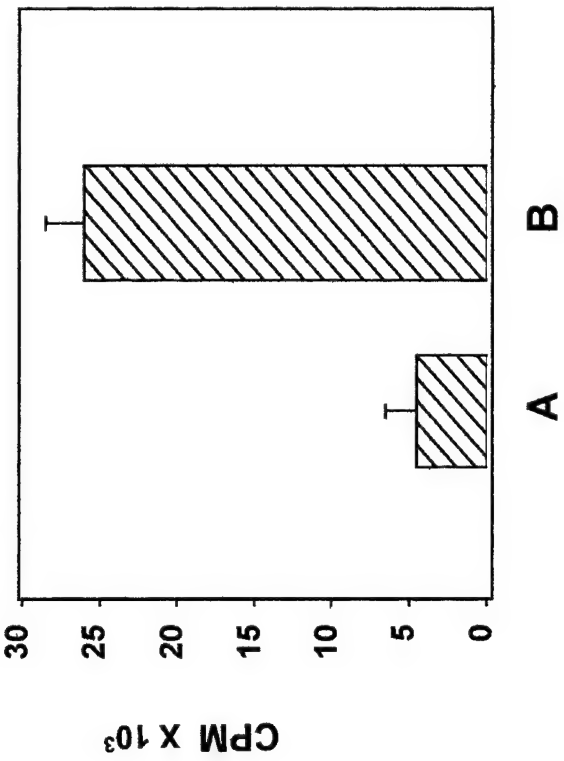
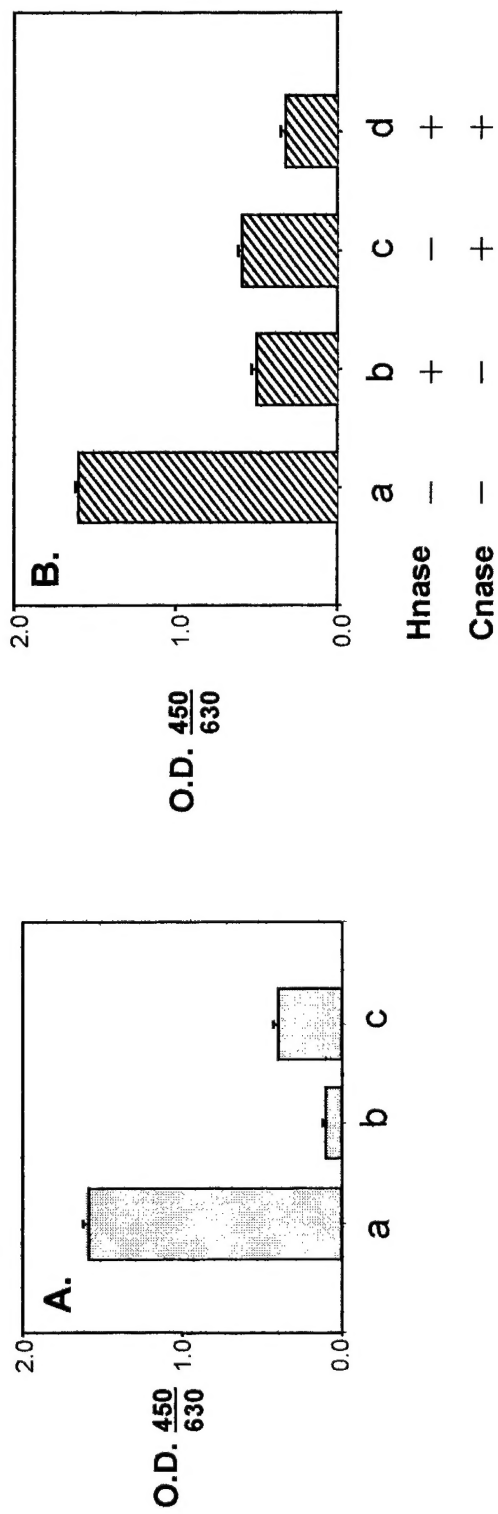


Fig. 3

Fig. 4



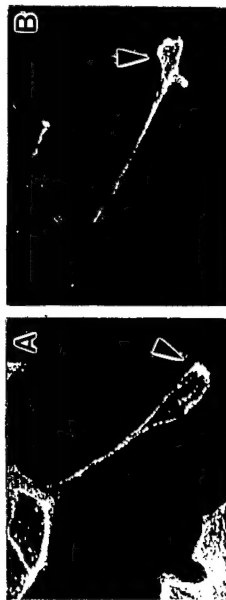


Fig. 5

Fig. 6

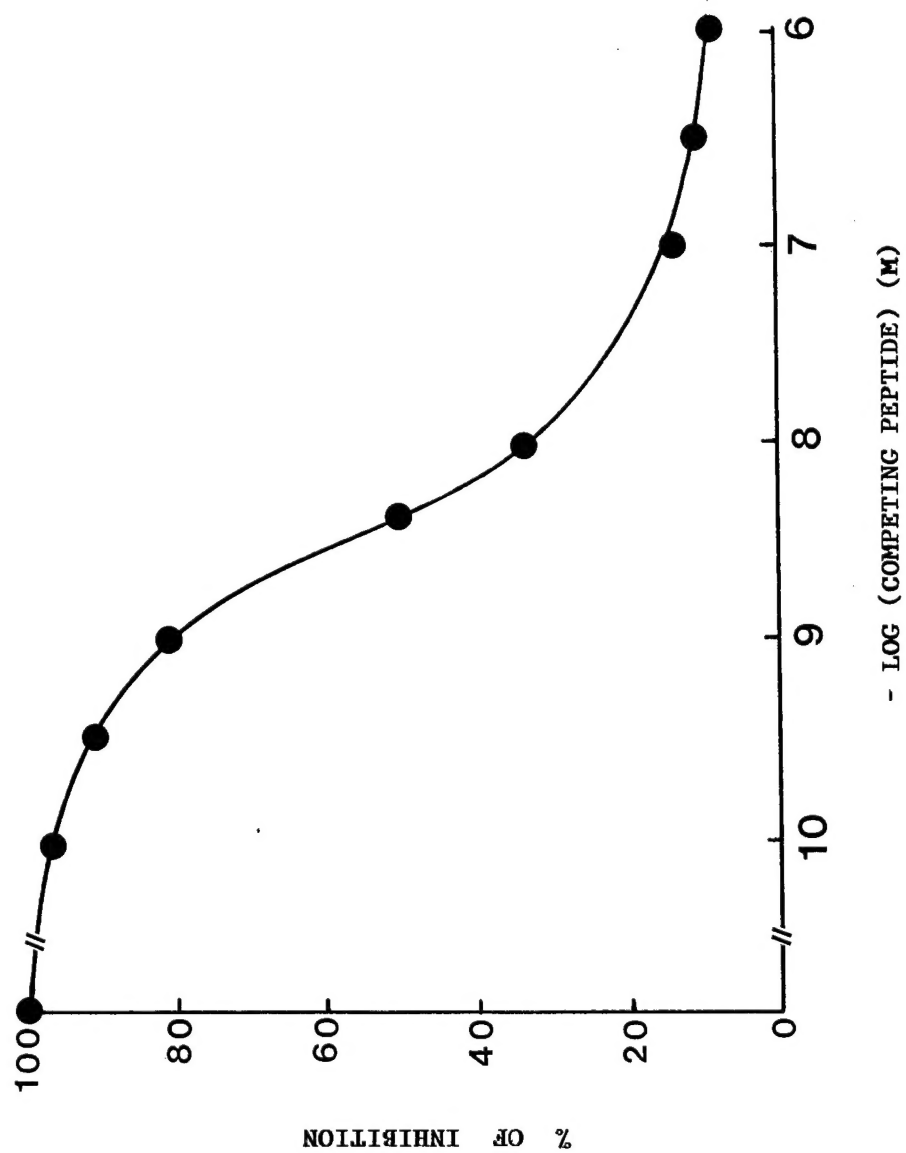


Fig. 7

